Identification of histamine receptor subtypes in skeletal myogenesis

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,GHQWLÀDWLRQRIKVWDPLQHUHFHSWRU VXEW\SHVLQVNHOHWDOPXVFOH

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[1]Department of Medicine, Institute of Clinical Medicine; [2]Department of Anatomy, University of Helsinki, Biomedicum 1, Helsinki 00029, Finland; [3]Department of Urology, Pediatric Urodynamic Center, Institute of Clinical Medicine, 7KH)LUVW$ÀOLDWHG+RVSLWDORI=KHQJ]KRX8QLYHUVLW\=KHQJ]KRX+HQDQ3U&KQD [4]6FKRRORI%LRORJLFDODQG%LRPHGLFDO6FLHQFHV'XUKDP8QLYHUVLW\'XUKDP'+/(8. [5]Institute of Pharmaceutical Chemistry, Goethe University, Frankfurt D-60438, Germany; [6]ORTON Orthopedic Hospital of the ORTON Foundation, Helsinki 00280; [7]COXA Hospital for Joint Replacement, Tampere 33520, Finland

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Abstract. To date, conventional and/or novel histamine receptors (HRs) have not been investigated in mouse skeletal myogenesis. Therefore, the present study aimed to investigate the HR-subtypes in skeletal myogenesis. The myogenesis of C2C12 skeletal myoblasts was evaluated using desmin, myogenin and myosin heavy chain (Myh) as early, intermediate and late differentiation markers, respectively. Reverse transcription-quantitative polymerase chain reaction and immunostaining were performed and the messenger RNA (mRNA) expression levels of the HR-subtypes and markers were determined. H_1R mRNA was found to be highly expressed in myoblasts at day 0; however, the expression levels were reduced as differentiation progressed. By contrast, H_2R mRNA expression remained constant, while H_3R mRNA expression increased by 28-, 103- and 198-fold at days 2, 4 and 6 compared with the baseline level (day 0), respectively. In addition, Myh expression increased by 7,718-, 94,487- and 286,288-fold on days 2, 4 and 6 compared with the baseline expression level (day 0). Weak positive staining of the cells for H_3R protein was observed on day 2, whereas highly positive staining was observed on days 4 and 6. HR expression during myogenesis was, in part, regulated by the stage of differentiation. These UHVXOWVDORQJZLWKUHYLRXVÀQGLQJVLQGLFDWHGSRVVLEOHLQYROYHPHQWRIKVXEW\SH ment of H_1R in the regulation of progenitor cell mitogenesis and of H_2R in the relaxation of acetylcholine-stimulated contraction of muscle cells, following the activation of professional histamine-producing cells, including mast cells. By contrast, H_3R may participate in the regulation of specialized myocyte functions, potentially by maintaining the relaxed state under WKHLQÁXHQFHRIFRQVWLWXWLYHO\KLJKDQGORZKLVWDPLQH concentrations, locally produced/released by non-professional histamine-producing cells.

Introduction

Histamine is a well-known biogenic and cationic amine, which is synthesized, stored and released by professional histamine-synthesizing cells. Mast cells, basophils and enterochromaffin cells are considered to be professional histamine-producing cells (1). Histamine is synthesized by histidine decarboxylase (HDC), which converts L-histidine to histamine (1). Histamine is released into and stored within storage granules, prior to regulated release (1). Following activation of professional histamine-producing cells, a burst release results in a transient high histamine concentration in the extracellular VSDFH7KHVHWUDQVLHQWKLVWDPLQHFRQFHQWUDWLRQV to stimulate the conventional histamine receptors, histamine receptor type 1 (H_1R; EC_{50} = 4.2] and histamine receptor type 2 (H_2R; EC_{50} = 4.3) (2). Smooth muscle cells (3,4), cardiomyocytes (5,6) and skeletal muscle tissue (7) express these conventional histamine receptors, which regulate cellular proliferation and the contraction state of the cells stimulated via the histamine/H_1R or H_2R axes (3-6).

$SUHYLRXVVWXGLHGWKHQRYHOKLVWDPLQHUHFHSWRU VXEW\SH ‘pro-form’ of HDC produced histamine, however, at a 100-1,000-fold lower rate compared with the typical enzyme isoform of the professional histamine-synthesizing cells (1). In non-professional histamine-producing cells, histamine is released into the cellular cytoplasm rather than being stored, and is therefore not subjected to regulated burst release (8). These cells contain organic cation transporters, which are equilibrative uniporters and transport the intracellularly synthesized

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Key words: myogenesis, differentiation, histamine receptor type 3, histamine

Table I. Primer sequences used in reverse transcription-quantitative polymerase chain reaction and the corresponding amplicon lengths.

Gene	Forward primer	Reverse primer	Length (bp)
Des	5'-GCCCTCAAGGGCACCAACGA-3'	5'-TTGCTCGGGGCTGGTTTCTCG-3'	297
Myog	5'-CCCAACCAGCGGCTGCCTAA-3'	5'-GTAGGGTCAGCCGCGAGCAA-3'	245
Myh2	5'-AGCTGCACCTTCTCGTTTGCCA-3'	5'-CGGTCAGGGTCGCTCCTGCT-3'	261
H_1R	5'-CACTGGAGGCTGCCCTTGTGC-3'	5'-CACCAGCAGGTTGAGGCCCAC-3'	167
H_2R	5'-TCCTAAGCGACCCGGTACAGC-3'	5'-ATGGAGACTGAGGCACTGCTGG-3'	208
H_3R	5'-TTCGAGCCTCCGCACCCAGAA-3'	5'-GGTCCAACGGCCGGTCAGC-3'	118
H_4R	5'-TGCTCAGGTCCCCTTGGCATTT-3'	5'-ACGTGAGGGATGTACAGAGGAATGG-3'	189
PBGD	5'-AAAGTGCCGTGGGAACCAGC-3'	5'-CAGCCACAGCCAGGACGATG-3'	156

Des, desmin; Myog, myogenin; Myh2, myosin heavy chain IIa; H_nR, histamine receptor type n (n=1-4); PBGD, porphobilinogen deaminase.

histamine from the non-professional histamine synthesizing cells along the histamine concentration gradient to the extracellular space (8). Histamine concentrations achieved in this manner DUHQRWVXIÀFLHQWWRVWLPXODWHFRQYHQWLRQDOK3 receptors. Therefore, this mechanism was hypothesized to represent an ancestral vestigium of a function that had become obsolete during phylogenesis. However, studies conducted within the last decade that focus on G-protein coupled receptors have revealed novel members of the histamine receptor family (2). These novel histamine receptors, histamine receptor type 3 (H_3R; S. $_i$ = 8.0) and histamine receptor type 4 ($H_4$5S. $_i$ = 8.2), have >10,000-fold JUHDWHUDIÀQLWIRUKVWDPLQHFRPSDUHGZLWKFRQYHQWLRQDO conventional receptors (2). In addition, the low basal levels of histamine produced by non-professional histamine-producing cells, including dendritic cells (9) and lymphocytes (10,11), KDYHEHHQGHPRQVWUDWHGWRHQDEOHWKHVHQRYHOKLJKDIÀQLW\ regulate cells equipped with these novel, high-DIÀQLW\KVWD mine receptors. The role of high histamine concentration in the regulation of muscle cell tone was investigated in previous studies (3-6). Studies using histamine receptor agonists and/or antagonists have suggested that novel histamine receptors may also be present and functional in the bronchial smooth muscle cells at least (3). However, to date, no studies indicating the presence of histamine receptors at the messenger RNA (mRNA) and protein level in myoblasts, myocytes or myotubes during skeletal myogenesis have been reported. Due to the presence and role of H_1R, H_2R and H_3R in the function of other muscle cell types, the present study aimed to assess whether striated muscle cells synthesize and express the histamine receptors, H_3R and H_4R. In addition, the current study investigated whether these receptors are developmentally regulated during myogenesis in association with various markers of myogenic maturation.

0DWHULDOVDQGPHWKRGV

Cell culture. The present study was approved by the institutional Medical Ethics Committee of the Institue of Clinical Medicine, University of Helsinki (Helsinki, Finland) and was performed in accordance with the 1983 Declaration of Helsinki. Mouse C2C12 myoblasts were obtained from the Turku Center for Biotechnology, University of Turku (Turku, Finland) (12), and maintained in growth medium FRPSULVLQJ'XOEHFFRV PRGLÀHG(DJOHV PHGLXP '(0 Lonza/BioWhittaker, Walkersville, MD, USA) supplemented with 10% fetal bovine serum (FBS; HyClone, DQG P0 /JOXWDPLQHQH]DW &LQDKPLGLÀ otics (100 U/ml penicillin and 100 µg/ml streptomycin; Lonza) 5% CO_2 atmosphere. The composition of the differentiation medium was similar to the growth medium, with the exception of FBS, which was reduced from 10% to 1%. The cells were passaged using trypsinization (0.5% trypsin in 0.5 mM EDTA; Gibco-BRL Life Technologies, Carlsbad, CA, USA)

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). To investigate the expression of histamine receptors during C2C12 myogenesis, 50,000 cells/well were seeded in 12-well plates (CellStar; Greiner Bio-One, Frickenhausen, Germany). The cells were initially grown in the medium was exchanged with differentiation medium to induce myogenesis. Total RNA was isolated from the cells at days 0, 2, 4 and 6 using an RNeasy Mini kit (Qiagen, Düsseldorf, Germany) according to the manufacturer's instructions. Total RNA (1 µg) was reverse transcribed using iScript cDNA Synthesis kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). RT-qPCR was performed with QJÀ VWDQGDUG1 XVLQJL46<%*Green Supermix (Bio-Rad Laboratories, Inc.) in an iCycler iQ5 Multicolor Real-Time PCR Detection system (Bio-Rad Laboratories, Inc.). Primers for mouse desmin (Des), myogenin (Myog), myosin heavy chain IIa (Myh2), H_1R, H_2R, H_3R, H_4R and porphobilinogen deaminase (PBGD) genes were designed using the National Center for Biotechnology Information Primer-Blast tool (Table I; http://www.ncbi.nlm.nih.gov/tools/primer-blast/; accessed: 01/03/2012). The mRNA copy numbers of the samples analyzed were determined in triplicate and normalized against the PBGD gene.

,PPXQRÀXRUHVFHQFHVWDLQLQJ C2C12 cells were seeded at $2x10^4$ cells/well in 24-well plates (CellStar) on coverslips and grown in growth medium for two days to reach 80% confluence, followed by culturing in differentiation

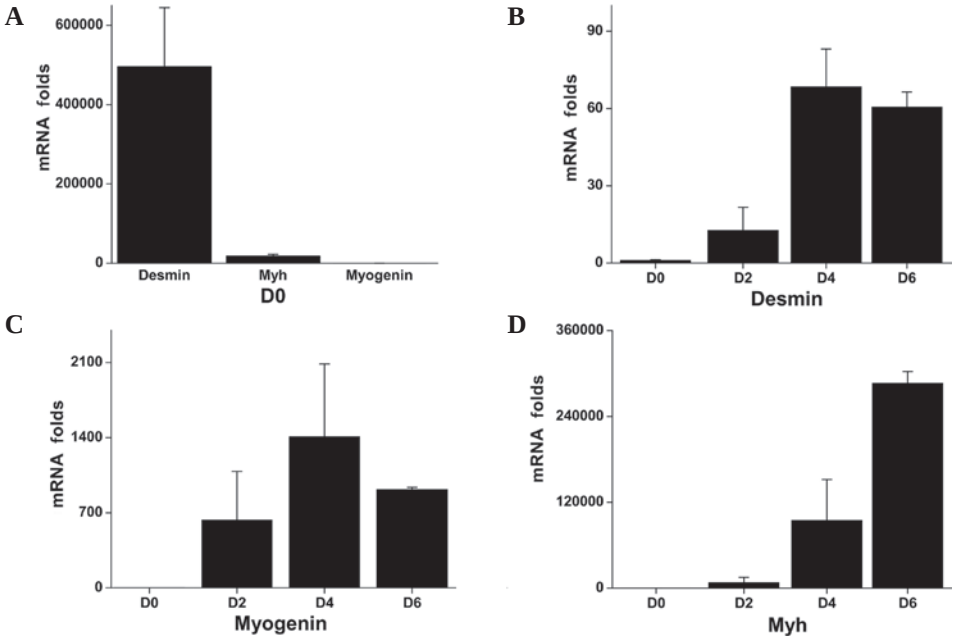


Figure 1. Myogenesis differentiation marker expression levels, determined by reverse transcription-quantitative polymerase chain reaction. (A) Relative expression levels of myogenic differentiation markers desmin (early marker), myogenin (intermediate marker) and Myh (late marker) in C2C12 cells at day 0, and relative expression level variations in (B) desmin, (C) myogenin and (D) Myh during myogenesis at days 2, 4 and 6. The data are expressed as the mean ± standard deviation. Myh, myosin heavy chain; mRNA, messenger RNA; D, day.

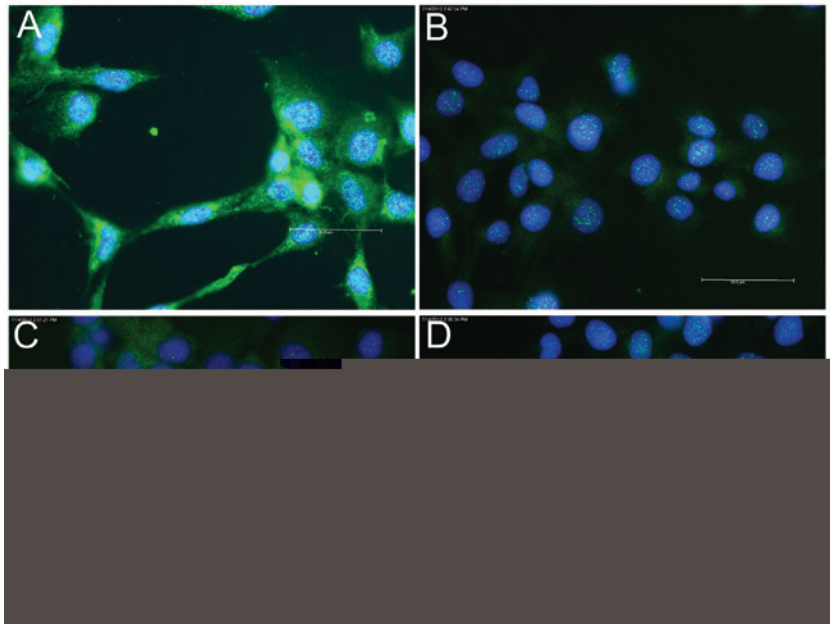
medium to induce myogenesis. Differentiated cells from GDV DQG ZHUH ¿[HG IRU PLQ LQ SDUDIRU maldehyde (Sigma-Aldrich, St. Louis, MO, USA) with phosphate-buffered saline (PBS; 10mM phosphate buffer, 140 mM saline; pH 7.4), washed three times in PBS (5 min HDFK WLPH DQG LQ 7ULWRQ 7 K UPR)LVK U6FLHQWL¿ Fair Lawn, NJ, USA)/PBS for 15 min to permeabilize the cells. Subsequently, the cells were cultured under the following conditions sequentially: i) 10% normal donkey serum (Jackson Immunoresearch Laboratories, Inc., West Grove, PA, USA) for 1 h; ii) 1 µg/POSRO FORQDO SHSWLGH DIÀQLW SXUL¿HG anti-human desmin (1:200), myogenin (1:400) or myosin heavy chain (Myh) immunoglobulin G (IgG; 1:400) antibodies (obtained from Dr John E. Erikson, University of Turku, Turku, Finland) (12), or rabbit anti-human H_3R polyclonal antibodies (1:1,000; LS-A476; MBL International, Woburn, 0$ 86$ DW & RYHUQLJK W DQG ZDVK HG WK HH WLPH V LQ 3%6 (5 min each time). Non-immune rabbit IgG (1:1,000; 1 µg/ml; R&D Systems, Minneapolis, MN, USA), was used at the same concentration as the primary antibodies as a negative staining control; iii) secondary antibody AlexaFluor®488-conjugated monoclonal donkey anti-rabbit IgG (1:400; Invitrogen Life Technologies, Carlsbad, CA, USA) in 0.1% bovine serum albumin (Sigma-Aldrich)-PBS for 1 h and washed three times in PBS (5 min each time); iv) DAPI dye (Sigma-Aldrich; 1:2,000 in distilled water) for 5 min. The coverslips were washed twice in PBS and distilled water for 10 min, prior to mounting with Vectashield medium (Vector Laboratories, Inc., Burlingame, CA, USA). Labeled slides were analyzed and photographed using a Leica DM 6000 B/0 ÁRUHVFHQFH microscope, with a motorized Leica XY-stage connected to a Leica DFC 420 digital camera, and analyzed using the Leica Application Suite Advanced Fluorescence 2.5.0.6735 software (Leica Microsystems GmbH, Wetzlar, Germany).

Statistical analysis. SPSS software, version 17.0 (SPSS, Inc., Chicago, IL, USA) was used to perform statistical analyses in addition to Matlab (MathWorks, Inc., Natick, MA, USA), which was used to perform the Mann-Whitney U test. All values are presented as the mean ± standard error of the mean. $P<0.05$ was considered to indicate a statistically VLJQL¿FDQW GLIIHUHQFH EHWZHHQ YDOXHV

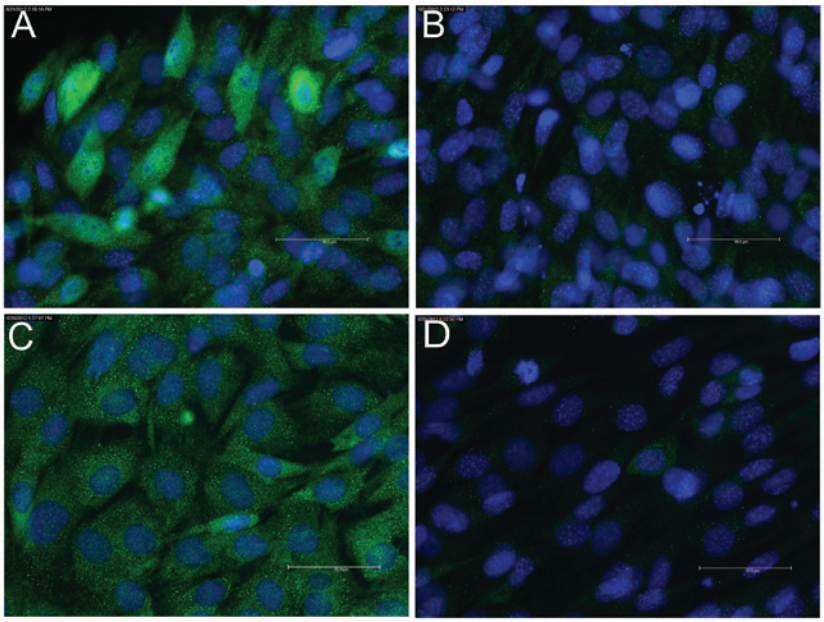
5HVXOWV

5HDO WLPH 4 3&5 RI & & FHOOU RT-qPCR was used to detect the mRNA expression levels of the early, intermediate and late myogenesis markers, desmin, myogenin and Myh2, respectively, during differentiation. On day 0, desmin was expressed in myoblasts at significantly higher levels compared with the myogenin or Myh (Fig. 1A). The desmin expression levels increased during myogenesis, reaching 12-, 68- and 60-fold over the baseline level (day 0), on days 2, 4 and 6, respectively (Fig. 1B). On day 0, the myogenin mRNA exxpression levels were low; however, the mRNA expression levels increased by 631-, 1,408- and 914-fold at days 2, 4 and 6, respectively (Fig. 1C). Desmin and myogenin expression levels peaked on day 4, whereas the expression of Myh, a late myogenesis marker, continued to increase over the entire study period, reaching 7,718-, 94,487- and 286,288-fold higher expression levels at days 2, 4 and 6, respectively, compared with the baseline level (Fig. 1D).

Indirect immunofluorescence staining of the myogenesis marker proteins revealed positive staining of the early marker, desmin, at day 0 (Fig. 2A); however, no staining was observed for the intermediate marker, myogenin (Fig. 2B), or the late marker, Myh (data not shown). On day 2, staining for myogenin was found to be positive (Fig. 3A), whereas staining for Myh remained negative (Fig. 3B). On



)LJXUH,PPXQRÁXRUHVFHQWVWDLQLQJRIWKHODWHPDUNHUPSLQ%PRJHQLQ& KVWDPLQHUHFHSWRUVH QHJDWLYHFRQWURO&RQIRFDOLPDJHV
C2C12 cells. Staining of the late myogenic marker myogenin heavy chain was negative (not shown). DAPI was used for nuclear counterstaining (blue color). Scale bar, 50 μm.



)LJXUH,PPXQRÁXRUHVFHQWVWDLQLQJRIWKHODWHPDUNHUPSLQ% PRVLQH& KVWDPLQHUHFHSWRUVH QHJDWLYHFRQWURO&RQIRFDOLPDJHV
differentiated C2C12 cells. DAPI was used for nuclear counterstaining (blue color). Scale bar, 50 μm.

days 4 (data not shown) and 6, positive staining for myogenin (Fig. 4A) and Myh (Fig. 4B) was detected.

([SUHVVLRQRI KVWDPLQHUHFHSWRUV was used to detect the mRNA expression levels of histamine receptors associated with the differentiation stages (Fig. 5). H_1R mRNA was found to be highly expressed in C2C12 myoblasts (day 0), whereas expression was decreased during the differentiation process (Fig. 5A and B). By day 6, the expression level decreased to ~25% of the baseline level (day 0). H_2R mRNA was also expressed in C2C12 cells and the expression levels remained relatively constant throughout the differentiation process (Fig. 5A and C). The expression of H_3R was found to be low in C2C12 myoblasts; however, following differentiation, the expression levels increased by 28-, 103- and 198-fold over the baseline level on days 2, 4 and 6, respectively (Fig. 5A and D). H_4R mRNA expression was not detected at any time-point.

,QGLUHFW LPPXQRÁXRUHVFHQFH VWDLQLQJ RI +$ during the myogenesis of C2C12 cells revealed almost negative staining at day 0 (Fig. 2C), weakly positive staining on day 2 (Fig 3C) and strongly positive staining on days 4 (data not shown) and 6 (Fig. 4C).